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Clinical, histological, immunohistochemical and genetic factors associated with measurable response of high-risk canine mast cell tumours to tyrosine kinase inhibitors

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Abstract. The aim of the present prospective-retrospective study was to evaluate the response of high-risk canine mast cell tumours (MCTs) to tyrosine kinase inhibitors (TKIs) and to correlate this with prognostic factors. A total of 24 dogs presented with macroscopic cutaneous MCTs at disease stage II or III, and therefore, at high-risk of associated mortality, were included in the study and treated with masitinib (n=20) or toceranib (n=4). A total of 12/24 dogs achieved an objective response and the overall survival (OS) for all subjects was 113 days. Dogs responding to treatment had a significant increase in OS compared to non-responders (146.5 days vs. 47 days, P=0.02). Internal tandem duplications in exon 11 of the c-kit gene were identified in 6/24 cases. Ki67, KIT immunolabelling and c-kit mutation did not provide information regarding prognosis or prediction of response to TKIs in this population. Initial response to TKIs appears to be the most reliable prognostic factor for survival duration.

Introduction

Mast cell tumour (MCT) is the most common cutaneous malignancy in dogs (1). In view of the wide variation in its biological behaviour, many prognostic factors have been proposed and evaluated in an attempt to improve decision making in the management of this neoplasm (2-4). Among the therapeutic approaches, surgery stands out as the optimal treatment offering the highest rate of cure for most low to intermediate grade MCTs (1,3,5). However, for high grade or biologically aggressive tumours, surgical benefit is limited and metastasis may occur in up to 90% of cases (2,3). Numerous drugs, including glucocorticoids, chemotherapeutic agents and tyrosine kinase inhibitors (TKIs) have been used for treatment of non-resectable MCTs, but the prognosis for such tumours remains guarded to poor (1,3,4). Tyrosine kinases (TKs) are enzymes located on the cell surface, cytoplasm or nucleus, that catalyze the transfer of phosphate groups from adenosine triphosphate molecules (ATP), leading to cellular signaling transmission. In cancer cells, several abnormalities may be found in specific protein kinases, which allows transduction of intracellular signals that ultimately will cause changes in gene transcription, increase cell proliferation, invasion and survival (6,7). Genetic and epigenetic changes can result in alteration in oncogenes or tumour suppressor genes expression leading to constitutively activated TKs , or abnormal TKs interactions (7-9).

Several molecular abnormalities have been identified and characterized in Veterinary Medicine, particularly in canine MCTs (10). Gain of-function mutations involving the KIT receptor and its pathway are considered relevant for the prognosis and treatment of MCT (11-14).

Dysregulation of several TKs have been found in different human cancers. Monoclonal antibodies like trastuzumab and cetuximab that respectively target HER-2 and EGFR TK receptors have been approved for human breast cancer (7,15). Imatinib mesylate is a small molecule TKI with a multi-target action towards KITr, PDGFR and Bcr-Abl protein. Imatinib is a well recognized and effective treatment for human gastro-intestinal stromal tumours and chronic myeloid leukemia (16). In veterinary medicine, imatinib was occasionally used in the treatment of canine MCT (17), and greatest effort was directed to the development ofsimilar TKI for veterinary use (7,10).

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Masitinib mesylate and toceranib phosphate, are TKIs licensed for use in dogs with non-resectable Grade II or III MCTS in Europe and the United States. They both act intracellularly in the protein kinases KITR and PDGFR α/β, where masitinib also operates in Lyn, Fyn and Lck (18), and toceranib in VGFR and Flt-3 (19). The action against multiple therapeutic targets, allows these molecules to interfere more effectively in the different pathways responsible for cancer progression (7). However, despite the development of such drugs and their increasing use in clinical practice, there is still a lack of established factors that can predict the response to treatment of canine MCTs toTKIs (4,10).

The objective of this study was to evaluate measurable responses of canine MCT to TKIs, correlating this with clinical, histopathological, imunohistochemical and genetic prognostic factors.

Materials and methods

Subject selection and treatment. This study included subjects retrospectively collected from the Queen's Veterinary School Hospital (QVSH) at the University of Cambridge (Cambridge, UK) (n=10), and prospectively enrolled from the Veterinary Hospital of the Universidade Federal de Minas Gerais (UFMG, Belo Horizonte, MG, Brazil)(n=14). The dogs were enrolled if presented with macroscopic cutaneous MCT and stage II, III or IV disease, considered to be at high-risk of MCT related death. For classification as a high-risk stage II, only a cytological diagnosis of certain metastasis, was accepted (20).

Incisional biopsies of primary tumours were performed and subjected to histological (Patnaik and Kiupel grading systems), immunohistochemical (Ki-67 and KITr) and genetic (*c-kit* oncogene) assessment. Clinical staging was performed by physical examination, abdominal ultrasound, fine needle aspiration and cytology of regional lymph nodes, satellite or distant skin lesion and suspected visceral lesions. Lymph node metastasis were identified, on fine needle aspirates (FNA) using cytological criteria previous published (20).

Dogs were treated with masitinib, at a dosage ranging from 8 to 12.5 mg/kg q 24 h or toceranib at a dosage of 2.5-2.7 mg/kg q 48 h. The concomitant use of prednisone or prednisolone, at an initial dosage of 40 mg/m^2, daily, (7-10 days), followed by a dosage of 25 mg/m^2, daily or every other day was often used, along with gastric acid inhibitors (omeprazole, ranitidine), for controlling paraneoplastic effects related to degranulation of mast cells.

Follow up information was collected from the subjects medical records or when necessary by telephone call conversation with the referring veterinary surgeon or the owner. Subjects which failed to comply with TKIs treatment or attendance during the clinical follow-up were excluded from this study.

This study was approved by the Ethics Committee on Animal Use (UFMG, protocol 384/2013) and Department's Ethics and Welfare Committee (University of Cambridge, protocol CR 138).

Histological analysis. The surgical specimens of the primary tumours were fixed in 10% formalin, cut in longitudinal sections for paraffin embedding, and 4 μm sections were mounted in glass and stained with hematoxylin-eosin and toluidine blue. Histopahological examination was performed by FC and RH, and tumour grading was defined through the systems proposed by Patnaik *et al* (21) and Kiupel *et al* (22).

Immunohistochemical analysis. Sections of 4 μm were cut from a representative block for each case and collected on gelatin-coated slides. The slides were deparaffinized and rehydrated in an alcohol series. Antigenal retrieval was performed with an antigen retrieval solution (Target Retrieval Solution Citrate pH 6, DakoCytomation, Glostrup, Denmark) under pressurized heat (20-25 mmHg, 125˚C/2 min). Endogenous peroxidase was blocked by immersion in 3% hydrogen peroxide and protein blockage (Thermo Scientific UltraVision™ Protein Block; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Primary antibodies CD117 (policlonal, 1:800; DakoCytomation, Glostrup, Denmark) and MIB-1 (monoclonal, 1:25; DakoCytomation) were incubated at 4˚C, for 16 h (overnight) for KITr and Ki-67 reactions, respectively. Secondary antibody (Advance HRP Link; DakoCytomation) was incubated in the humidity chamber for 30 min and the reaction was amplified by the polymer (Advance HRP Enzyme; DakoCytomation). The reaction was revealed with the chromogen 3,3-diaminobenzidinetetrahydrochloride (Liquid DAB + SubstratChromogen System; DakoCytomation) and stained with Harris hematoxylin.

The immunolabelling pattern for KITr was evaluated, by counting membrane, focal or difuse cytoplasmic immunoexpression (KIT patterns I, II or III, respectively) in 100 mast cells at a x40 magnification. Each MCT was assigned with the highest staining pattern present in at least 10% of the neoplastic cell population or present in large clusters of neoplastic cells within the tumour, as described by Kiupel *et al* (23). Ki-67 value was determined as the percentage of positive nuclei in at least 500 neoplastic cells in 3-5 high power fields (x40 magnification). Every nucleus with evidence of immune labelling was considered positive for Ki-67. This approach was described by Scase *et al* (24). Previously tested canine MCT samples were used as positive control for KITr and Ki-67, and negative controls were obtained by replacing the primary antibody by normal serum.

Screening of mutations in the c-kit oncogene. The polymerase chain reaction (PCR) for amplification of the fragment of interest in the *c-kit* oncogene, was performed by Progen, in Vetpat Laboratory (Campinas, SP, Brazil), from the DNA extraction in paraffin embedded tumour, by the proteinase K method. The primers used in the bleaching of the reaction were designed with the help of the BLAST software (Basic Local Alignment Search Tool®, NCBI) and manufactured by Invitrogen (São Paulo, SP, Brazil), as *c-kit* forward: 5'-ATC TGTCTCTCTTTTCTCCCCC-3' (sense) and *c-kit* reverse: 5'-TGGGGTTCCCTAAAGTCATTGT-3' (antisense). The product generated by these pair of primers had 225 bp in the absence of mutations (native *c-kit*). Reactions were prepared and planned in a GenPro thermocycler (BIOER Technology), with a maintenance at 95˚C for five min, then 30 cycles of 94˚C for 45 sec for denaturation of DNA strands, 63˚C for 45 sec to pairing and annealing of primers and 72˚C for one minute

to extension, to be finally maintained at 72˚C for ten min for molecular stabilization. The amplified material was separated by electrophoresis at 100V, with free amperage. Canine healthy skin samples and milique water were used as positive and negative controls, respectively.

Assessment of response and toxicity. Tumour response to TKI was based on measurements of the primary tumour and all target lesions (including metastatic lymph nodes) before and two-weeks after starting treatment, as recommended by the Response Evaluation Criteria for Solid Tumours (RECIST, v.1.0) (25). Complete response (CR) was defined as a complete disappearance of the mass(es), partial response (PR) was defined as at least 30% reduction in size, stable disease between 20% reduction and 20% increase in size, progressive disease was defined as an increase in size of the mass of more than 20%. Overall response rate (ORR) was calculated based on the total number of subjects that achieved complete and partial response (CR+PR). The disease-free interval (DFI), for subjects who achieved complete remission and overall survival (OS) for all subjects were calculated from the start of TKI administration. Cytology was used to confirm the diagnosis in case of progressive disease and appearance of new lesions. Side effects related to the use of TKI were recorded according to Veterinary Cooperative Oncology Group-Common Terminology Criteria for Adverse Events (VCOG-CTCAE v.1.1) (26).

Statistical analysis. Statistical analysis was performed using GraphPad Prism (v.6.01). A matrix correlation was built through Spearmann test for searching association between prognostic factors and overall survival. DFI and OS were estimated through Kaplan-Meier curve and the log-rank test of Cox-Mantel was used to compare the curves, according to prognostic factors. $P<0.05$ was considered to indicate a statistically significant difference. Significant correlations were considered strong when they occurred in over than 49% of the studied population ($r>0.07$), moderate, as occurred in 9-49% ($0.3<r<0.7$), and weak, when they occurred in less than 9% of the population ($r<0.3$).

Results

A total of 24 dogs were included in this study (Table I). Fourteen cases were enrolled prospectively, from the Veterinary Hospital, UFMG and 10 cases were retrospectively included, identified from medical records of subjects treated at the QVSH, University of Cambridge. Tyrosine kinase inhibitors were used as first line therapy in 11 dogs and as a rescue treament in 13 dogs. All except one subject received concomitant prednisone (n=13, all from UFMG) or prednisolone (n=10, from QVSH). Sixteen subjects had received previous chemotherapeutic agents including: lomustine (n=9), vinblastine (n=4), lomustine followed by chlorambucil (n=2), lomustine followed by vinblastine (n=1). Toceranib was used instead of masitinib in four subjects.

An objective response was obtained in 12/24 subjects (50%), seven of which had CR (29%) and five PR (21%) as shown in Figs. 1 and 2, respectively. Stable (n=4; 17%) or progressive disease (n=8; 33%) was observed in 12 subjects (50%). One subject developed partial remission with masitinib, as a first line therapy, resulting in the tumour becoming resectable. Surgery was performed and the subject continued masitinib treatment with a DFI of 86 days, and an OS of 288 days (144 days after surgery).

The overall survival time for all subjects in this study was 113 days but DFI and OS for subjects who achieved CR was 140 and 164 days. In a matrix correlation only the initial response to TKIs was associated with OS (P=0,03; r_S=0,578). As shown in Fig. 3, subjects who achieved measurable responses during the first weeks of treatment (n=12) reached the median at 146 days, while those who remained with stable or progressive disease (n=12) reached the median at 47 days (P=0.02).

Eleven subjects were treated with TKIs as a first line treatment, but 81.8% (9/11) of these, were treated only after post surgical recurrence of the tumour. The ORR for tumours treated with TKI as first line treatment was 54.5% (6/11). Thirteen dogs received TKIs as a second line treatment and 69,2% (9/13) of these had previous surgery as well. The ORR for tumours treated with TKI as a second line treatment was 46,2% (6/13). The difference in ORR between the two groups of subjects treated with TKIs as a first or second line treatment was not statistically significant. There was also no significant difference in DFI and OS for the same two groups of subjects, however a tendency for significance in OS was found between the first line treatment compared to the second line treatment group (160 and 103 days, respectively; P=0,2). Similarily, there was no difference in ORR between subjects treated on the first presentation of MCT or after post surgical recurrence of the tumour, however a tendency for significance in OS was found between non-recurrent and recurrent MCTs (123 and 66 days, respectively; P=0,09).

Clinical staging was also not statistically related to prognosis, and subjects in stage II (n=6) and III (n=17), reached a median OS of 130 and 123 days, respectively (P=0,8). There was also no influence of histological grade, mitotic index (1-60 mitotic figures in 10 high-power fields) and Ki-67 value (5,4-46,0%) in OS of these subjects.

Abnormalities in KIT expression were identified in 17/24 (71%) MCTs, 12 with KIT II-pattern and four with a KIT III-pattern, but there was also no correlation with OS. Nevertheless, objective responses (CR+PR) were obtained in 28% (2/7), 54% (7/13) and 75% (3/4) of subjects whose tumours presented with KIT expression pattern I, II and III, respectively, although the number was not appropriate for a contingency analysis. Duplications in exon 11 of the *c-kit* gene were identified in 6/24 subjects (24%). Of these, measurable responses were observed in 4/6 (67%). A similar rate of response was found for subjects without any identified mutations, through the elected method (8/18, 44% of response to TKI). There was also no difference in OS, according to the mutational status in the exon 11 of the *c-kit* oncogene.

Positive correlations were found between mitotic index and both grading systems (P=0,009; r_S=0,523 for Patnaik grading system; P=0,001; r_S=0,617 for Kiupel grading system), KITr pattern and Patnaik grading system (P<0,00001; r_S=0,676) and both grading systems (P=0,0006; r_S=0,650). Ki67 was not correlated with MI or Patnaik and Kiupel grade.

Side effects were relatively common and are reported in Table II. One dog developed severe illness after

Table I. Clinical, histopahological, immunohistochemical and genetic features of 24 dogs submitted to treatment with tyrosine-kinase inhibitors for treatment of measurable disease.

N	Breed	Age (months)	Staging	Grade (Patnaik/ Kiupel)	Mitotic index	Ki-67 (%)	KITr	c-kit oncogene exon 11 mutational status	Previous treatment	Clinical response	Follow-up	Disease-free interval	Overall survival
01[a] (M)	Sharpei	72	III	Grade 2/ high grade	6	7.0	KIT I	Native	Prednisone, lomustine	CR	Euthanasia due to disease progression	40	133
02[a] (T)	French Bulldog	48	III	Grade 3/ high grade	20	33.0	KIT III	Native	Prednisone, vimblastine	CR	Natural death due to disease progression	124	131
03[a] (T)	Crossbreed	59	III	Grade 3/ high grade	60	28.7	KIT II	Native	Prednisone, lomustine	PD	Natural death due to disease progression	-	30
04 (M)	French Bulldog	35	III	Grade 3/ high grade	5	19.0	KIT III	Native	Prednisone, lomustine	PR	Euthanasia due to disease progression	-	103
05[a] (M)	Schnauzer	158	III	Grade 2/ low grade	3	26.0	KIT I	Native	Prednisone	PD	Euthanasia due to disease progression	-	49
06 (M)	Crossbreed	115	III	Grade 2/ low grade	2	13.0	KIT I	Native	Prednisone, lomustine	SD	Euthanasia due to disease progression	-	123
07[a] (T)	Cocker spaniel	133	III	Grade 3/ high grade	2	22.0	KIT III	Native	Prednisone, lomustine	SD	Euthanasia due to disease progression	-	125
08[a] (M)	Pinshcer	144	III	Grade 2/ high grade	4	13.0	KIT I	ITD	Prednisone	CR	Natural death due to disease progression	140	164
09 (M)	Crossbreed	123	III	Grade 2/ low grade	3	29.0	KIT II	ITD	Prednisone, lomustine	CR	Still alive but with signs of disease progression	240	280
10[a] (M)	Pinscher	132	III	Grade 3/ high grade	4	13.0	KIT II	Native	Prednisone	SD	Still alive	-	208
11[a] (M)	Crossbreed	162	II	Grade 3/ high-grade	41	14.6	KIT II	Native	Prednisone, lomustine	PD	Euthanasia due to disease progression	-	47
12[a] (T)	Schnauzer	156	IV	Grade 2/ high grade	33	34.0	KIT I	Native	Prednisolone, vimblastine	PD	Euthanasia due to disease progression	-	15
13 (M)	Schnauzer	144	III	Grade 2/ low grade	1	28.0	KIT I	ITD	Prednisone,	PD	Euthanasia due to disease progression	-	12
14[a] (M)	Pinscher	120	III	Grade 2/ low grade	4	22.0	KIT II	ITD	Prednisone, lomustine, chlorambucil	PD	Euthanasia due to disease progression	-	42
15 (M)	Sharpei	120	III	Grade 3/ high grade	44	46.0	KIT II	Native	Prednisolone, lomustine, vimblastine	CR	Euthanasia due to disease progression	400	427

Table I. Continued.

N	Breed	Age (months)	Staging	Grade (Patnaik/ Kiupel)	Mitotic index	Ki-67 (%)	KITr	c-kit oncogene exon 11 mutational status	Previous treatment	Clinical response	Follow-up	Disease-free interval	Overall survival
16[a] (M)	Jack Russel Terrier	140	III	Grade 3/ high grade	15	9.0	KIT II	ITD	Prednisolone, vimblastine	PR	Euthanasia due to disease progression	-	57
17[a] (M)	Labrador	48	II	Grade 2/ low grade	2	6.3	KIT II	Native	Prednisolone	SD	Euthanasia due to disease progression	-	161
18[a] (M)	Boxer	60	II	Grade 2/ high grade	2	15.3	KIT II	Native	Prednisolone	PR	Euthanasia due to disease progression	-	101
19[a] (M)	Labrador	117	II	Grade 2/ low grade	2	6.0	KIT II	Native	Prednisolone	PR	Euthanasia due to disease progression	-	66
20[a] (M)	Dogue de Bordeaux	29	II	Grade 3/ high grade	28	37.8	KIT III	ITD	Prednisolone	CR	Natural death due to disease progression	114	203
21[a] (M)	Greyhound	132	II	Grade 3/ high grade	4	9.3	KIT II	Native	Prednisolone	CR	Euthanasia due to disease progression	52	160
22[a] (M)	Border Collie	145	III	Grade 2/ low grade	5	12.4	KIT I	Native	Prednisolone	PD	Euthanasia due to disease progression	-	47
23[a] (M)	Poodle	36	III	Grade 2/ high grade	14	5.4	KIT II	Native	Prednisolone, vimblastine	PD PR	Euthanasia due to disease progression	-	23
24 (M)	Labrador	105	III	Grade 3/ high grade	12	6.8	KIT II	Native	-		Euthanasia due to disease progression	- 86	288 141

[a]Recurrent tumour. ITD, internal tandem duplication; M, mastinib; T, toceranib; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Table II. Adverse side effects observed in 24 dogs with advanced staged mast cell tumours treated with tyrosine kinase inhibitors (23 were also treated with glucocorticoids).

Adverse side effect	Grade	Frequency (%)
Anaemia	Grade 2	1/24 (4.2)
	Grade 4	1/24 (4.2)
Thrombocytopenia	Grade 4	1/24 (4.2)
Neutropenia	Grade 1	16/24 (66.7)
ALP increase	Grade 1	12/24 (50)
ALT increase	Grade 1	1/24 (4.2)
	Grade 2	2/24 (8.3)
Azotemia	Grade 2	1/24 (4.2)
Proteinuria	Grade 1	1/24 (4.2)
(increase urine	Grade 3	1/24 (4.2)
protein/creatinine ratio)	Grade 4	1/24 (4.2)
Hypoalbuminaemia	Grade 4	1/24 (4.2)

ALP, alkaline phosphatase; ALT, alanine transferase.

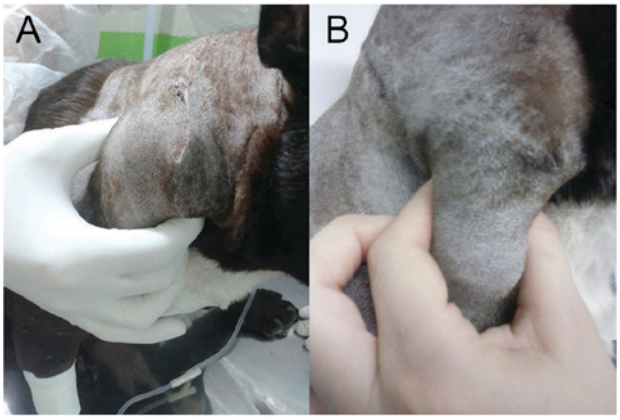

Figure 1. French Bulldog presenting with (A) a mast cell tumour metastasis on cervical superficial lymph node, (B) but with complete remission after 12 days of treatment with masitinib mesylate.

133 days of masitinib. The subject presented with a grade 4 non-regenerative anaemia with concomitant thrombocytopenia, grade 2 azotemia and grade 4 proteinuria resulting in hypoalbuminemia/ascites (nephrotic syndrome). The dog was treated with total blood transfusion and fluidtherapy and the drug was suspended, but despite the subject's recovery, tumour recurrence was noted 28 days later and the dog was euthanized.

Discussion

In this study, as previously reported by Smrkovski *et al* (27), a 50% ORR was observed in dogs with unresectable MCTs, treated with TKIs.

The OS of dogs in this study was lower in comparison to reports of Smrkvoski *et al* (27) and Hahn *et al* (28). Three main hypotheses might explain this difference: Firstly, our study included a high number of subjects with post surgical recurrent MCTs (18/24) which showed reduced survival rates compared to non recurrent MCT, althought this was not statistical significant. A poor outcome is historically reported for recurrent MCTs with related death rates reaching 86-100% of cases as reported by Patnaik *et al* (21); Secondly, TKIs were administered after failure of chemotherapy in over half of these subjects. As shown by Hahn *et al* (28), better responses were obtained when mastinib mesylate was used as a first line treatment. However, in our study, no differences were seen in OS in subjects treated as a first or second line treatment with TKIs. A third and most likely hypothesis is that the concomitant use of glucocorticoids might have impaired a favourable and prolonged response to TKIs. The mechanisms involved in tumour resistance to TKIs are still largely unknown, but appear to involve abnormalities in genes responsible for the synthesis of other areas of the targeted proteins, or development of alternative cellular pathways (29). Another mechanism of resistance is the overexpression of ABC transporters (30). Imatinib and dasitinib are TKIs similar to masitinib in its mechanism of action and they are substrates of the ABC transporters, such as P-glycoprotein (P-gp, ABCB1) and breast cancer resistance protein (BCRP, ABCG2), both induced by the administration of glucocorticoids (30). Masitinib is also a P-gp substrate and P-gp overexpression can increase the resistance to masitinib (31). However, multitarget TKIs similar to toceranib, as sunitinib, have been found to inhibit the ABC (32,33). The authors and collaborators found a significant increase in survival in subjects treated with masitinib alone compared to subjects treated with masitinib in combination with prednisolone (data still not published). The efficacy of mastinib could be reduced by the development of a rapid drug resistance caused by the induction of P-gp, from previous or concurrent prednisolone treatment, while toceranib could or could not be affected. In our study only four subjects were treated with toceranib and prednisolone, too few to allow any conclusion. Further studies are needed to evaluate the benefit of adding corticosteroids to masitinib or toceranib.

In this study, one subject received adjuvant therapy with masitinib, once this TKI resulted in partial response of its previous unresectable disease, making it resectable. This subject reached an OS of 288 days from the beginning of neoadjuvant treatment with masitinib, superior to the median obtained in this study (113 days). This observation could suggest that TKIs responses in the treatment of gross disease may also be useful in the adjuvant scenario. In the presence of minimal residual disease, a reduced development of tumour resistance and even a synergism with other therapeutic approaches could be hypothesized. New clinical trials are required to evaluate the response of canine MCT to these drugs in the adjuvant setting.

In this case series, including dogs with advanced staged disease, the initial response to TKIs was the most significant prognostic factor, as previously reported by Smrkovski *et al* (27) and Grant *et al* (34). In contrast, histological grade, mitotic index, Ki-67 value, KITr pattern and even the mutational status in exon 11 of the *c-kit* oncogene had no impact on OS for these subjects. Increased

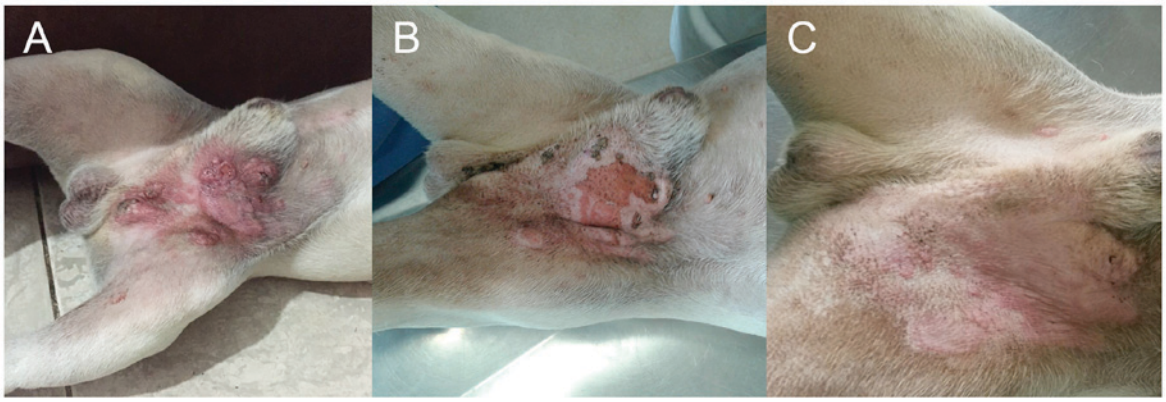

Figure 2. French Bulldog presenting a cutaneous mast cell tumour in the prepuce region with (A) favorable response to masitinib mesylate, resulting in partial remission in (B) 10 days and (C) 30 days. Note the persistence of satellite nodules on the skin near the prepuce (C).

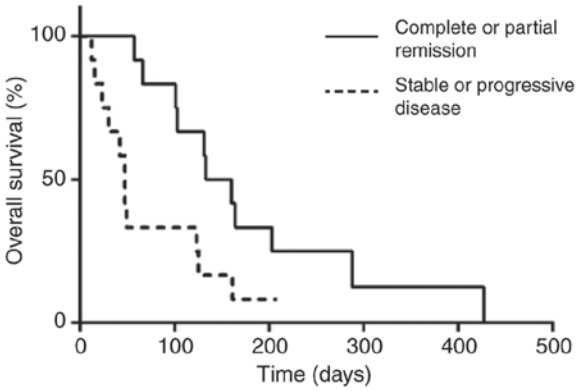

Figure 3. Graphical representation of overall survival for 24 subjects with advanced-stage, macroscopic canine mast cell tumour, treated with tyrosine kinase inhibitors, in accordance with the initial response to treatment (Md=146.5 days for those who presented partial or complete response and Md=47 days for those with stable or progressive disease; P=0.02). Graph Pad Prism v. 6.01.

response rate was found in subjects with II and III KITr staining patterns and in the presence of ITD in the exon 11 of *c-kit* oncogene, however due to the low number in each subcategory the statistical significance could not be evaluated. The prognostic value of KITr immunelabelling pattern has been evaluated in some studies and although Kiupel *et al* (2004) showed that the KITr immunelabelling pattern could be a prognostic factor for canine MCT (23), this was not confirmed in more recent studies (35,36). The relevance of *c-kit* mutational status, as a predictor for TKI response was suggested in older studies (17,19,28). We found an increase response rate in samples arboring *c-kit* mutations compared with samples with absent mutations, however the number of cases was too small to draw any significant conclusion.

The main limitations of this study were the relatively low number of samples and the heterogenicity of the subjects and type of treatment used, however this is often a common problem in studies of canine MCTs. Genetic assessment of exon 11 was performed using PCR analysis rather than genetic sequencing, so point mutations in the exon 11, could not be assessed. Primers applied in this study were limited only to the exon 11, but whereas there might be *c-kit* activating mutations in other loci, like exons 2, 5, 6, 7, 8, 9 and 15, (14), these are not proven, at the current state of our knowledge, to be of prognostic or predictive significance.

Although the number of cases in this study was small, there was no correlation between mitotic index and Ki-67 value, which differs from the study conducted by Berlato *et al* (37). However a moderate correlation was found between mitotic index and both grading systems, Patnaik's grading system and KITr pattern. As expected and previously demonstrated by Giantin *et al* (35), both grading systems were also moderately correlated with each other.

Masitinib and toceranib are generally well tolerated in dogs, although mild and self-limiting side effects may occur. However, clinical-pathological abnormalities should always be monitored, once severe side effects may occur, like non regenerative anaemia and moderate to severe proteinuria, as seen in our study and also by Miller *et al* (38).

In conclusion, TKIs can be effective in the treatment of macroscopic advanced staged canine MCTs. Nevertheless, there is lack of factors that could strongly predict the response to treatment. Similar to other studies, we found that the initial response to treatment is the only reliable prognostic factor for those subjects regardless of theclinical stage, histological grade and mitotic index. Nevertheless, history of recurrent MCTs and previous chemotherapeutic agents may reduce response rate. As found in our preliminary results, concomitant use of glucocorticoids may impair the response to TKIs and possibly induce early TKI resistance resulting in reduced OS. Differently to other similar papers published before all samples were evaluated for Ki-67 value, immunohistochemical pattern of KITr and even the mutational status in exon 11 of the *c-kit* oncogene. Ki67, KITr immunostaining and *c-kit* mutation did not give any further relevant informations regarding prognosis and or in the prediction of response to TKIs in the cohort of high-risk MCTs examined, although expression of KIT II and III might result in higher response rate.

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